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IL10 low-frequency variants in Behçet's disease patients

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Abstract

Aim: To explain the missing heritability after the genome-wide association studies era, sequencing studies allow the identification of low-frequency variants with a stronger effect on disease risk. Common variants in the interleukin 10 gene (*IL10*) have been consistently associated with Behçet's disease (BD) and the goal of this study is to investigate the role of low-frequency *IL10* variants in BD susceptibility.

Methods: To identify *IL10* low-frequency variants, a discovery group of 50 Portuguese BD patients were Sanger-sequenced in a 7.7 kb genomic region encompassing the complete *IL10* gene, 0.9 kb upstream and 2 kb downstream, and two conserved regions in the putative promoter. To assess if the novel variants are BD- and/or Portuguese-specific, they were assayed in an additional group of BD patients (26 Portuguese and 964 Iranian) and controls (104 Portuguese and 823 Iranian).

Results: Rare *IL10* coding variants were not detected in BD patients, but we identified 28 known single nucleotide polymorphisms with minor allele frequencies ranging from 0.010 to 0.390, and five novel non-coding variants in five heterozygous cases. ss836185595, located in the *IL10* 3′ untranslated region, was also detected in one Iranian control individual and therefore is not specific to BD. The remaining novel *IL10* variants (ss836185596 and ss836185602 in intron 3, ss836185598 and ss836185604 in the putative promoter region) were not found in the replication dataset.

Conclusion: This study highlights the importance of screening the whole gene and regulatory regions when searching for novel variants associated with complex diseases, and the need to develop bioinformatics tools to predict the functional impact of non-coding variants and statistical tests which incorporate these predictions.

Key words: Behçet's disease, IL10, low-frequency variants, sequencing, susceptibility.

INTRODUCTION

Behçet's disease (BD) is an immune-mediated inflammatory vasculitis with a large clinical spectrum, including oral aphthae, genital ulcerations, uveitis, as well as vascular, neurological, articular, pulmonary and gastrointestinal manifestations.[1] In complex diseases such as

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BD, genome-wide association studies (GWAS) have provided new insights into disease physiopathology through the identification of common genetic variants (minor allele frequencies [MAF] greater than 5%) associated with disease susceptibility.[2,3] However, these single nucleotide polymorphisms (SNPs) typically confer a small disease risk and only explain a modest portion of the overall genetic variance.[4] In addition, many SNPs captured by GWAS seem to be in linkage disequilibrium with the causal variant rather than to be causal themselves, and most of the associated genetic variants do not have any known or obvious functions.[5,6] Moreover, low-frequency ($1\% \leq MAF < 5\%$) and rare variants ($MAF < 1\%$) are not sufficiently frequent to be captured by GWAS and current efforts to discover these variants by sequencing have focused on genes within regions implicated by GWAS.[7]

In addition to the well-established human leukoctye antigen (*HLA*) locus, common polymorphisms in interleukin (*IL23R–IL12RB2*) and *IL10* have been recently associated with BD in two independent GWAS in Turkish and Japanese datasets[2,3] and replicated in an Iranian sample[8] and in a recent meta-analysis.[9] IL10 has a central role in the suppression of inflammatory cytokines inhibiting the costimulatory activity of macrophages for T-cell and natural killer (NK)-cell activation. IL10 deficiencies add to the group of primary immunodeficiencies and result in severe deregulation of the immune system.[10] Interestingly, elevated IL10 levels have been observed in the sera and mucocutaneous lesions of BD patients.[11] It is likely that relative levels of cytokine production may be important in the determination of disease onset, progression and outcome, and therefore we hereby chose to focus our attention on *IL10*.

To further investigate the role of low-frequency *IL10* variants in BD susceptibility, we sequenced the complete *IL10* gene and neighboring genomic regions, including two well-conserved regions in the putative promoter. The existence and association with BD of identified novel variants was then assessed in a second independent dataset.

PATIENTS AND METHODS

Study subjects

The 76 Portuguese BD patients were recruited throughout Portugal and 104 Portuguese controls were selected among blood bank donors or healthy volunteers using the same evaluation procedures used for the cases; individuals were included as controls when negative for BD, any other rheumatologic or autoimmune disorder, oral and genital aphthosis. The Iranian dataset included 1787 unrelated subjects (964 BD cases and 823 controls) recruited at the Rheumatology Research Center of Shariati Hospital (Tehran University of Medical Sciences, Tehran, Iran). The diagnosis of BD was made according to the revised International Criteria for Behçet's Disease.[1] Patients with age at onset of BD after 60 years were excluded. The clinical and demographic features of the participants were obtained by medical interview at the time of blood sampling and inspection of medical records. All participants were informed of the study, provided informed written consent and the study was conducted according to the Declaration of Helsinki. This study received ethics approval from the ethics committees in Portugal (Hospital Infante D. Pedro and Instituto Português de Reumatologia) and Iran (Tehran University for Medical Sciences).

Sample preparation

Blood samples were drawn into ethylenediaminetetraacetic acid (EDTA) tubes and stored at −20°C until genomic DNA was extracted using QIAamp® DNA Blood Maxi kits (Qiagen, Germantown, MD, USA) or a salting out procedure and diluted in Tris–EDTA (TE) buffer. The concentrations of extracted DNA were determined by Nanodrop using absorbance readings at 260 nm and the DNAs were then stored at 4°C.

Sequencing

To sequence the *IL10* open reading frame and two conserved promoter regions, 13 primer sets (Table S1) were designed using the PRIMER3 software (http://bioinfo.ut.ee/primer3/). Polymerase chain reaction (PCR) amplification was carried out in 12.5 μL reaction mixtures containing: 25 ng of genomic DNA, 0.004 μg/μL of each primer, 1 × PCR reaction buffer, 0.2 mmol/L deoxynucleotide triphosphate (dNTP), 1.5 mmol/L $MgCl_2$, and 0.05 U/μL $Vent_R$® DNA polymerase (New England Biolabs, Ipswich, MA, USA). DNA amplification was performed on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using a touchdown protocol (94°C for 3 min, 15 cycles of [94°C for 30 sec, starting at 70°C decreasing 1°C/cycle for 30 sec, 72°C for 1 min], 30 cycles of [94°C for 10 sec, 55°C for 45 sec, 72°C for 1 min], 72°C for 7 min) to amplify all regions except PCR 4. For PCR 4 (forward primer sequence: GTTTGGAGCCTTCCCTCTCT and reverse primer sequence: AAGCCTGACCACGCTTTCTA), we used a GC-Rich PCR system kit (Roche Molecular Biochemicals, Mannheim, Germany) in a 20 μL reaction containing 25 ng of genomic DNA, 0.2 pmol/μL of each

primer, 0.2 mmol/L dNTPs, 5 mol/L GC-Rich resolution solution, 1 × GC-Rich PCR reaction buffer and 0.04 U/50 μL GC-Rich Enzyme Mix. The PCR program was as follows: 95°C for 3 min, 30 cycles of (95°C for 30 sec, 58°C for 30 sec, 72°C for 45 sec), 72°C for 7 min. PCR products were separated on a 3% agarose gel, visualized under UV light after staining with Gel Red (Biotium, Hayward, CA, USA), and purified using ExoSAP-IT enzyme (USB Corporation, Cleveland, OH, USA) to remove unincorporated primers and dNTPs.

A 10 μL sequencing reaction was prepared with 90 ng of PCR product, 1 × reaction buffer, 1 × BigDye v3.1 Cycle Sequencing Kit (Applied Biosystems) and 0.32 pmol/μL of primer (forward or reverse). The sequencing reaction was then subjected to thermal cycling (96°C for 1 min, followed by 25 cycles of (96°C for 10 sec, 50°C for 5 sec, 60°C for 110 sec), followed by a rapid thermal ramp to 4°C) and run on an automated sequencer (ABI 3730XL, Applied Biosystems) in the DNA Sequencing Service from the Genomics Unit at the Instituto Gulbenkian de Ciência in Portugal. Sequencher 4.10.1 software demo version (Gene Codes Corporation, Ann Arbor, MI, USA) was used to analyze the raw sequence data.

Genotyping

Five variants were genotyped using a Sequenom iPlex assay (Sequenom, San Diego, CA, USA) according to the manufacturer's protocol, with detection in a Sequenom MassArray K2 platform. The primer sequences (Table S2) were designed using Sequenom MassARRAY Assay Design software version 3.0 and the genotyping assay was performed using the Genotyping Service from the Genomics Unit at the Instituto Gulbenkian de Ciência, Portugal. Extensive quality control was performed with the use of eight HapMap controls of diverse ethnic affiliation, along with sample duplication within and across plates, a Mendelian inheritance check in three large pedigrees, and a call rate for each SNP > 90%. Genotype determinations were performed with the investigators blinded to the affection status (i.e., case or control). Individual DNA samples with genotyping success rate across all SNPs < 85% were also excluded.

Transcription factor binding sites

The Integrated Regulation from ENCODE (Transcription Factor ChIP-seq from ENCODE) and the ENCODE Transcription Factor Binding (Transcription Factor ChIP-seq Uniform Peaks from ENCODE/Analysis, ENCODE March 2012 Freeze) tracks on the UCSC genome browser (http://genome.ucsc.edu/) were used to determine if the identified novel variants are located in putative transcription factor binding sites.

RESULTS

IL10 variant discovery

To identify novel low-frequency variants in *IL10* associated with BD, we first sequenced a 7.7 kb genomic region encompassing the complete *IL10* gene plus 0.9 kb upstream and 2 kb downstream of the gene (Chr1: 206.938.978 to Chr1: 206.946.691 in the GRCh37/hg19 assembly), as well as two conserved regions in the putative *IL10* promoter (826 bp at Chr1:206.950.949–206.951.775 and 845 bp at Chr1:206.957.492–206.958.337). This variant discovery was performed by Sanger sequencing of 50 BD Portuguese patients (100 chromosomes), which allows the detection of variants with MAF > 1%. The principal demographic and clinical features of the subjects in the Portuguese dataset are shown in Table 1. The Portuguese cases and controls are age-matched and all patients presented recurrent oral aphthosis. Other common manifestations included genital aphthosis (89.4%), skin (80.2%), ocular (45.9%), joint (77.3%), neurological (20.3%), vascular (13.9%), gastrointestinal (16.2%) and cardiac (5.5%) involvement.

In these 50 BD patients, we did not detect rare *IL10* coding variants, but we identified 28 known SNPs with MAF ranging from 0.010 to 0.390, and five previously undescribed single nucleotide variants (SNVs) in a heterozygous state in five BD patients (Table 2). The novel variants are located in the *IL10* 3′ untranslated region (ss836185595), in intron 3 (ss836185596 and ss836185602), and in the putative *IL10* promoter region (ss836185598 and ss836185604).

Association of novel SNVs with BD

To assess if these five novel low-frequency *IL10* variants are specific to BD patients and/or to the Portuguese population, we genotyped them in an additional Portuguese sample (26 cases and 104 controls) and in an Iranian dataset (964 cases and 823 controls) with the demographic and clinical characteristics shown in Table 1. ss836185595 was detected in one Iranian control individual (Table 2) and therefore constitutes a new rare variant found in different populations and not specific to BD patients. The remaining four SNVs (ss836185596, ss836185602, ss836185598 and ss836185604) were not found in this second Portuguese dataset or in the Iranian cases and controls (Table 2). ENCODE data annotated on the UCSC

Table 1 Main demographic and clinical features of Behçet's disease (BD) patients and controls in the Portuguese and Iranian datasets

Characteristic	Portuguese controls	Portuguese BD cases	Iranian controls	Iranian BD cases
No. of subjects	104	76	823	964
Sex, male, no. (%)	57 (54.8)	18 (23.7)	345 (41.9)	506 (52.5)
Age at examination, mean ± SD (years)	36.4 ± 13.6	36.3 ± 12.0	40.4 ± 11.9	39.1 ± 10.9
Clinical symptom, no./total assessed (%)				
Oral aphthosis	0/104 (0)	76/76 (100)	0/823 (0)	953/964 (98.9)
Genital aphthosis	0/104 (0)	68/76 (89.4)	0/823 (0)	603/964 (62.6)
Skin lesions		61/76 (80.2)		534/964 (55.4)
Pseudofolliculitis		50/76 (65.8)		405/964 (42.0)
Erythema nodosum		32/76 (42.1)		209/964 (21.7)
Skin aphthosis		7/76 (9.2)		30/964 (3.1)
Ophthalmologic manifestations		34/74 (45.9)		577/964 (59.9)
Anterior uveitis		20/74 (27.0)		416/964 (43.2)
Posterior uveitis		13/74 (17.6)		490/964 (50.8)
Retinal vasculitis		3/74 (4.1)		340/964 (35.3)
Joint manifestations		58/75 (77.3)		296/964 (30.7)
Arthralgia		51/75 (68.0)		137/964 (14.2)
Arthritis		21/75 (28.0)		185/964 (19.1)
Ankylosing spondylitis		1/75 (1.3)		18/964 (1.9)
Neurologic manifestations		15/74 (20.3)		61/964 (6.3)
Vascular involvement		10/72 (13.9)		51/964 (5.3)
Gastrointestinal manifestations		12/74 (16.2)		39/964 (4.0)
Epididymitis		2/73 (2.7)		21/964 (2.2)
Cardiac involvement		4/73 (5.5)		6/964 (0.6)
Pleuropulmonary involvement		2/72 (2.8)		6/964 (0.6)
Pathergy phenomenon		19/41 (46.3)		437/948 (46.1)
Family history of BD		11/71 (15.5)		85/959 (8.9)

genome browser showed that the reference sequences of these four novel SNVs lie in transcription factor binding sites (ss836185596: Pol2; ss836185602: NFKB, STAT3, TBP, Oct-2, POU2F2, Pol2, and Pol2–4H8; ss836185598: Pol2, NFKB, TCF12, p300, BATF, EBF1_[C-8], SP1, Oct-2, POU2F2, PAX5-N19, Sin3Ak-20, PAX5-C20, BCLAF1_[M33-P5B11], MEF2A, IRF4_[M-17], BCL11A, Egr-1, ZEB1_[SC-25388], YY1; ss836185604: NFKB, BCLAF1_[M33-P5B11], MEF2A, BCL11A, Egr-1, YY1 and ELF1_[SC-631]). However, using this tool, we cannot predict if the rare alleles of these variants affect the binding affinities of the transcription factors.

DISCUSSION

We identified four novel *IL10* non-coding variants in four Portuguese BD patients which were absent in Portuguese controls and in a large dataset of Iranian BD cases and controls. Even though the approaches were different (e.g., whole gene sequencing *vs.* exonic sequencing), our results are consistent with those from a recent report by Kirino *et al.*[7] where deep exonic resequencing of *IL10* (in 766 patients and 768 matched controls of Japanese or Turkish origin, distributed over 32 DNA pools) led to the identification of five non-synonymous rare mutations (G15R, M40V, R124Q, H127R, K315E). A meta-analysis of the associations (in a dataset of 528 Japanese BD cases and 586 controls and in a dataset of 1933 Turkish BD cases and 1872 controls) showed that these five rare mutations were not collectively associated with BD in any of the three burden tests assayed.[7] These authors did not screen the intronic regions of *IL10* and therefore it is unclear whether there would be an over-representation of non-coding variants among their patients when compared to controls.

Since the novel low-frequency variants are not in coding regions, we cannot predict their potential functional impact using algorithms such as SIFT[12] or PolyPhen2,[13] and therefore we cannot test their association with BD using currently available tests such as the C-alpha test.[14] Our work highlights the need to develop bioinformatics and statistical tools capable of estimating the impact of

Table 2 *IL10* variants identified in Behçet's disease (BD) patients

Variant ID	Position (base pairs)†	Localization	Alleles	Minor allele frequency			
				Portuguese BD cases (n = 76)	Portuguese controls (n = 104)	Iranian BD cases (n = 964)	Iranian controls (n = 823)
rs3024505	206939904	Downstream	A/G	0.120 (A)‡	–	–	–
rs3024502	206940310	Downstream	C/T	0.360 (T)‡	–	–	–
rs3024500	206940831	Downstream	A/G	0.350 (G)‡	–	–	–
rs3024498	206941529	3′ UTR	C/T	0.380 (C)‡	–	–	–
rs3024510	206941636	3′ UTR	A/T	0.010 (A)‡	–	–	–
rs3024496	206941864	3′ UTR	A/G	0.320 (G)‡	–	–	–
ss836185595	206941923	3′ UTR	C/T	0.007 (T)	0.000 (T)	0.000 (T)	0.001 (T)
rs45587633	206942187	Intron 4	C/T	0.010 (T)‡	–	–	–
rs45611331	206942244	Intron 4	C/T	0.020 (T)‡	–	–	–
rs3024495	206942413	Intron 4	C/T	0.120 (T)‡	–	–	–
rs3024509	206943297	Intron 3 (boundary)	A/G	0.060 (G)‡	–	–	–
ss836185596	206943312	Intron 3 (boundary)	T/C	0.007 (C)	0.000 (C)	0.000 (C)	0.000 (C)
rs3024508	206943410	Intron 3	A/C	0.010 (C)‡	–	–	–
rs1878672	206943713	Intron 3	C/G	0.370 (C)‡	–	–	–
rs3024493	206943968	Intron 3	A/C	0.140 (A)‡	–	–	–
rs3024507	206944077	Intron 3	C/T	0.020 (T)‡	–	–	–
ss836185602	206944078	Intron 3	G/A	0.013 (A)	0.000 (A)	0.000 (A)	0.000 (A)
rs3024492	206944112	Intron 3	A/T	0.120 (A)‡	–	–	–
rs1554286	206944233	Intron 3 (boundary)	A/G	0.320 (A)‡	–	–	–
rs1518111	206944645	Intron 2 (boundary)	C/T	0.350 (T)‡	–	–	–
rs1518110	206944861	Intron 1	A/C	0.390 (A)‡	–	–	–
rs3021094	206944952	Intron 1	G/T	0.150 (G)‡	–	–	–
rs3024491	206945046	Intron 1	A/C	0.360 (A)‡	–	–	–
rs3024490	206945311	Intron 1	A/C	0.370 (A)‡	–	–	–
rs2222202	206945381	Intron 1	A/G	0.350 (G)‡	–	–	–
rs5743625	206946115	Promoter	A/G	0.010 (A)‡	–	–	–
rs1800872	206946407	Promoter	G/T	0.340 (T)‡	–	–	–
rs56299498	206946413	Promoter	C/T	0.010 (T)‡	–	–	–
rs1800895	206946472	Promoter	C/T	0.020 (T)‡	–	–	–
rs12122923	206951397	Promoter	C/T	0.070 (T)‡	–	–	–
rs17015767	206951398	Promoter	C/G	0.150 (C)‡	–	–	-
ss836185598	206957911	Intergenic	C/T	0.007 (T)	0.000 (T)	0.000 (T)	0.000 (T)
ss836185604	206958076	Intergenic	C/T	0.013 (T)	0.000 (T)	0.000 (T)	0.000 (T)

The five variants identified for the first time in this study are highlighted in bold. †The base pair positions refer to the GRCh37/hg19 release. ‡Minor allele frequencies in the 50 Portuguese BD patients used in the mutation screen. UTR, untranslated region.

non-coding variants (e.g., in splice sites, in introns or in the promoter region) and to incorporate these predictions into an association test.

The approach to perform mutation discovery in BD patients only relies on the assumptions that cases carry deleterious mutations and controls do not harbor protective alleles. It is a cost-effective strategy when non-synonymous variations are not found in patients, as it avoids screening the controls. The lack of variant discovery in the controls may introduce a bias in our study as we may have missed protective variants, but this possibility is unlikely and not supported by the study by Kirino *et al.*[7]

The new variants we identified may play a role in gene expression regulation through modification of transcription factor binding sites or DNA methylation

patterns, especially those located in the promoter region. At this stage, we lack information on the specific impact of these new variants in *IL10* function to decipher whether these polymorphisms contribute or not to BD. Thus, gene expression studies and functional analyses of *IL10* variants, as well as the identification of the genetic/environmental factors, are needed to reinforce the potential implication of this gene in the susceptibility to BD.

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DISCLOSURE STATEMENT

The authors have declared no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Sequences of primers used to amplify *IL10* and promoter regions.

Table S2 Sequences of primers used for genotyping the new variants.